INHIBITION BY DIGITONIN OF BOVINE HEART MUSCLE DPNH-CYTOCHROME c REDUCTASE AND ITS SPECIFIC REVERSAL BY THE TOCOPHEROLS*

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The primary mechanism of action of vitamin E (the tocopherols) in the living cell has not yet been determined (see reviews by Boyer, 1960; Vasington et al., 1960, Symposium on Vitamin E, 1962). Among the prominent possibilities for a key metabolic role of vitamin E are (a) a protective action as an intracellular antioxidant, (b) an unknown function in nucleic acid metabolism, and (c) as a component, either directly in electron transport (and/or in phosphorylation reactions), or indirectly, perhaps as a structural agent of the cytochrome c reductase portion of the terminal respiratory chain in mammalian striated muscle.

In support of the latter proposal that vitamin E may be an essential component of the cytochrome c reductase of mammalian striated muscle, the present communication reports that the activity of purified DPNH-cytochrome c reductase of bovine heart muscle (and of rat skeletal muscle) is markedly inhibited by digitonin, and is specifically and fully restored by the tocopherols (α, β, γ and δ -forms). All other substances tried including other fat soluble vitamins, various glycerides, fatty acid esters, synthetic antioxidants, steroids, and coenzyme Q failed to prevent or reverse digitonin inhibition.

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For these experiments various fractions of DPNH-cytochrome c reductase, succinate-cytochrome c reductase and cytochrome oxidase from bovine heart muscle and rat skeletal muscle were used, and their enzymatic activities measured spectrophotometrically in 0.1 M phosphate buffer, pH 7.5, essentially as described (Lehman and Nason, 1956). DPNH-ferricyanide and indophenol-reductase activities, as well as succinate-ferricyanide and indophenol-reductase activities, were determined spectrophotometrically using potassium ferricyanide (10^{-3}M) or trichlorophenol indophenol (7×10^{-4}M) as the electron acceptor in place of cytochrome c (in 0.1 M Tris buffer at pH 7.9 in the case of indophenol-reductase activity) and measuring the rate of decrease of optical density at 420 mμ and 635 mμ, respectively.

The data of Table I show that digitonin inhibited the activity of a 100-fold purified bovine heart muscle DPNH-cytochrome c reductase about 80 percent, and that this inhibition could be largely or entirely prevented by the addition of either α , β , γ or δ -tocopherol. Moreover, the immediate reversal, as well as prevention, of the inhibition has also been observed by adding the tocopherols to enzymes that have been kept for as long as several weeks with digitonin. The restored activity of DPNH-cytochrome c reductase, like that of the uninhibited enzyme, is extremely sensitive to antimycin A (about 0.004 micrograms of the antibiotic per ml reaction mixture causing 80 percent or more inhibition). None of the tocopherol derivatives tested, which included the esters α-tocopheryl succinate, α-tocopheryl phosphate, α-tocopheryl polyethylene glycol 1000 succinate, and α-tocopheryl acetate as well as tocopheryl quinone and its corresponding hydroquinone, were effective. Coenzyme Q_{10}, plastoquinone and vitamin K_1 also failed to restore activity. On a few rare occasions, however, coenzyme Q_{10} reversed digitonin inhibition but this could not be repeated. The apparent partial effectiveness of added menadione can be attributed to the latter's capacity to act as an artificial electron carrier enroute to cytochrome c via a different pathway. This is evident from the fact that the resulting enzyme activity under these circumstances is insensitive to antimycin A.

Table I - Effect of Various Lipids in Preventing Digitonin Inhibition of Purified Bovine Heart Muscle DPNH- and Succinate-Cytochrome c Reductases

Additions*	ΔA550 per 2' x 10^3 DPNH Reductase Digitonin**		Succinate Reductase	
		+	-	+
Control	118	21	137	5
Plus α-tocopherol	145	107	108	114
β-tocopherol	150	95	123	103
γ-tocopherol	146	79	120	110
δ-tocopherol	137	109	117	102
α-tocopheryl acetate	100	15	101	12
α-tocopheryl succ.(10μg,50μg)	21	14	102	96
α-tocopheryl phosphate	63	20	96	220
α-tocopheryl quinone	86	15	127	6
α-tocopheryl hydroquinone	62	17	114	19
Coenzyme Q_{10}	105	25	113	-4
Plastoquinone	119	28	-	-
Vitamin K_1	87	15	122	11
Menadione	105	62Δ	141	78Δ
Vitamin A alcohol (2μg,50μg)	55	15	60	-31
n-butyl stearate	70	13	104	16
Tripalmitin	70	11	101	3
Lecithin	54	27	145	155
Oleic Acid (0.5μg,50μg)	64	11	12	7
Cholesterol	66	13	103	15
estradiol (1μg,50μg)	60	15	115	-5
Tween 80 (.005μg,50μg)	52	11	119	172
Santoquin (10^{-6}M)	90	18	99	7

For the DPNH-cytochrome c reductase the reaction was started at zero time by the addition of 1.15 μmoles DPNH to a mixture containing about 3 μg of enzyme protein (5th gel eluate, Lehman and Nason, 1956), .1 ml 2% aqueous cytochrome c, 0.1 ml 5 x 10^{-2}M KCN, and 0.1 M phosphate buffer, pH 7.5 to give a final volume of 1.0 ml. For the succinate-cytochrome c reductase the reaction mixture was the same except that 0.1 ml 0.1 M sodium succinate and 22 μg of enzyme protein (Fraction III, Lehman and Nason, 1956) were used in place of DPNH and the 5th gel eluate, respectively.*

*Indicated substances were added at a final concentration of 50 μg (suspended in 0.2% aqueous albumin) per ml reaction mixture unles otherwise noted. Where two concentrations are shown the first applies to the DPN system and the second to the succinate system. Santoquin was added in ethanol solution.

**180 μg and 240 μg Digitonin (suspended in 15% ethanol, 0.2% albumin) per ml reaction mixture were used in the DPN and succinate systems, respectively.

Δinsensitive to antimycin A.

All other substances tested including selenite, selenate, manganous and cadmium ions (final concentrations ranging from 10^{-8} to 10^{-3}M), lipoic acid, butter, the fatty acid esters

n-butyl stearate and methyl palmitate, and the triglycerides, tripalmitin, tristearin and lipide "cofactor" (Donaldson, et al., 1958) had no preventative effect. In addition, the steroids cholesterol and dihydrocholesterol, the steroid hormones estrone, pregnandiol and estradiol, the synthetic antioxidants nordihydroguaiaretic acid, santoquin, dibutyl p-cresol, santoflex, amyl hydroquinone, diphenyl-p-phenylene diamine, and propylparasept, and the detergents tween 80, triton X-100, and decanal where ineffective against digitonin inhibition. Lecithin and several of the fatty acids such as palmitic, stearic, lauric, oleic and caprylic acids were also ineffective, and in fact were significantly inhibitory as were tween 80 and triton X-100, vitamins A and D, cholesterol, a number of fatty acid esters and triglycerides, certain steroid hormones, tocopheryl hydroquinone and the above esters of α-tocopherol other than tocopheryl acetate. Table I includes examples from most of the above classes of substances, indicating their typical inability to prevent digitonin inhibition. The data of Table I also illustrate the inability of these substances, except for the tocopherols, to reverse digitonin inhibition during the total reaction time of approximately three minutes. Similar results have been obtained using the DPNH-cytochrome c reductase of rat skeletal muscle.

Aside from the addition of the tocopherols, the only other treatment which reversed the inhibitory action of digitonin was a several-fold dilution of the inhibited enzyme. The reversible restoration by dilution is presumably due to a dissociation of digitonin from the enzyme. It very likely accounts for the fact that the inhibition of cytochrome c reductase by digitonin (which is often used to fragment and "solubilize" the mitochondrial terminal respiratory chain) has gone undetected until now, for in

most cases digitonin when employed as a "solubilizing" agent, is added to concentrated enzyme preparations, which must subsequently be diluted for purposes of assay.

Digitonin is also inhibitory to the succinate-cytochrome c reductase system of bovine heart muscle (and of rat muscle). However, in contrast to the case of DPNH-cytochrome c reductase, inhibition can be prevented or reversed not only by α, β, γ - and δ -tocopherols, but by several other substances as well. As shown in Table I, α-tocopheryl succinate, α-tocopheryl phosphate, α-tocopheryl polyethylene glycol 1000 succinate (but not α-tocopheryl acetate), lecithin and tween 80 can also prevent and reverse the digitonin inhibition, probably because of their known surface active properties. All other substances tested including selenate, selenite, several metal ions, the antioxidants and steroids failed to activate the inhibited enzyme.

Examination of the steady state oxidized difference spectra (not shown) of the bovine heart muscle DPNH-and succinate-cytochrome c reductases in the model 14 Cary spectrophotometer using DPNH or succinate as electron donors showed that the addition of digitonin resulted in an increased absorption peak of cytochrome b and a decreased absorption of the α-peaks of cytochromes c and a_3, indicating the site of inhibition of digitonin to be between cytochromes b and c, the same general region where antimycin A also inhibits.

When indophenol or ferricyanide was used as the electron acceptor in place of cytochrome c, the DPN enzyme was not inhibited by digitonin, whereas the succinate-ferricyanide system was. Inhibition of the latter system could be offset by tocopherol. Of the other cardiac glycosides and aglycones tested digoxin, gitalin, digitogenin, digitoxigenin, and gitoxigenin inhibited the DPNH-cytochrome c reductase system while the

succinate-cytochrome c reductase was affected only by digitonin and its aglycone digitogenin. In these instances added α-tocopherol reversed the inhibitory effect. Gitoxin, reserpine and ouabain had no effect on either system.

It is worth noting that in preliminary experiments the addition of digitonin to an aqueous tocopherol-bovine serum albumin suspension results in the sedimentation of 80 to 95 per cent of the α-, β, γ and δ -tocopherols, probably reflecting its known property to form molecular compounds with phenols. It has also been found to precipitate similar suspensions of cholesterol, coenzyme Q, and vitamin K_1 (but not those of n-butyl stearate and vitamin A). The specific effect of the tocopherols in reversing digitonin inhibition of DPNH-cytochrome c reductase, therefore, cannot be attributed to a dissociation of digitonin from the enzyme by precipitation, since several of the above-mentioned substances would have been expected to do the same.

Finally, in contrast to its inhibitory effect on the cytochrome c reductases, digitonin is markedly stimulatory to the cytochrome oxidase of bovine heart muscle and rat skeletal muscle, increasing activity as much as four-fold. The stimulatory effect on cytochrome oxidase can be prevented in full, or in part, by a variety of substances including the tocopherols, α-tocopheryl succinate, α-tocopheryl phosphate, α-tocopheryl polyethylene glycol 1000 succinate, α-tocopheryl hydroquinone, lipid "cofactor," vitamin A acetate, vitamin D_2, methyl palmitate, tristearin, palmitic acid, lauric acid, monolein, linoleic acid, distearin, capric acid, lecithin, estradiol and tween 80 (not shown). The stimulatory effect of digitonin on cytochrome oxidase does not account for the observed inhibition of cytochrome c reductase, since the increased cytochrome oxidase activity, like that of the endogenous activity, is cyanide-sensitive. Sufficient cyanide

(5×10^{-3M} final concentration) is routinely included in the system to inhibit entirely the cytochrome oxidase activity in the absence or presence of digitonin.

It is possible that digitonin inhibits cytochrome reductase by a depolymerizing action, perhaps as a result of combining with tocopherol which is known to be present in various mitochondrial terminal electron transport fractions including the Keilin-Hartree heart-muscle preparations and purified DPNH-cytochrome c reductase (Nason and Vasington, 1959; Crane et al., 1959; Slater, 1961; Hatefi et al., 1961). This aspect of the problem is now being investigated.

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